Molecular epidemiology of Cryptosporidium spp. and Giardia spp. in mussels (Mytilus californianus) and California sea lions (Zalophus californianus) from central California

Running title: Cryptosporidium and Giardia in mussels and sea lions

A.D. Adell¹, W.A. Smith²*, K. Shapiro¹, A. Melli¹ and P.A. Conrad¹,²

¹Pathology, Microbiology and Immunology Department, School of Veterinary Medicine, One Shields Ave, University of California, Davis, CA 95616.
²One Health Institute, School of Veterinary Medicine, 1089 Veterinary Medicine Dr, University of California, Davis, CA, 95616.
²Escuela de Medicina Veterinaria, Facultad de Ecología y Recursos Naturales, Universidad Andres Bello, Republica 440, 8370251 Santiago, Chile

*Corresponding Author:
Tel: 1-530-754 0119
Email: wasmith@ucdavis.edu

Keywords: Cryptosporidium, Giardia, PCR, Mussel, California Sea Lion, Molecular Epidemiology
Abstract

Cryptosporidium and Giardia are of public health importance, with recognized transmission through recreational waters. Therefore, both can contaminate marine waters and shellfish, with potential to infect marine mammals in nearshore ecosystems. A 2-year study was conducted to evaluate the presence of Cryptosporidium and Giardia in mussels located at two distinct coastal areas in California: a) land runoff plume sites, and b) near sea lion haul-out sites, as well as in feces of California sea lions (CSL) (Zalophus californianus) using direct fluorescent antibody (DFA) detection methods and polymerase chain reaction (PCR) with sequence analysis. In this study, 961 individual mussel hemolymph samples, 54 aliquots of pooled mussel tissue, and 303 CSL fecal samples were screened. Giardia duodenalis assemblages B and D were detected in hemolymph from mussels collected near two land runoff plume sites (Santa Rosa Creek and Carmel River) and assemblages C and D were detected in hemolymph from mussels collected near a sea lion haul-out site (White Rock). These results suggest that mussels are being contaminated by protozoa from terrestrial runoff and/or were shed in the feces of CSL. Furthermore, low numbers of oocysts and cysts morphologically similar to Cryptosporidium and Giardia, respectively, were detected in CSL fecal samples; suggesting that CSL could be a source and a host of protozoan parasites in coastal environments. The results of this study showed that Cryptosporidium and Giardia spp. from the feces of...
terrestrial animals and CSL can contaminate mussels and coastal
environments.

Introduction

Cryptosporidium and Giardia spp. are zoonotic waterborne pathogens
known to be released into the environment through human and animal fecal
contamination (1-3). Previous studies have shown that these fecal protozoal
parasites and other enteric pathogens are transported via freshwater
sources to estuarine ecosystems in California (4-6). In coastal areas this
'pathogen pollution' may lead to contamination of surface waters and
shellfish, which can serve as a source of infection for humans and marine
mammals.

Cryptosporidium oocysts and Giardia cysts are ideally suited for
transmission through recreational waters because they can be excreted in
large quantities by animals and humans (7-10) and are immediately
infectious upon fecal excretion (3) even at a low doses (7, 8).

Cryptosporidium spp. are particularly problematic because oocysts may not
be removed by all water filtration processes due to their small size (5
microns). In addition, a fully efficacious drug treatment for cryptosporidiosis
remains elusive (3). Cryptosporidium oocysts and Giardia cysts can survive
for long periods of time in environmental waters, resist chlorine-based
treatments, and can be concentrated and remain infectious in shellfish (1, 2, 11-14).

California sea lions (CSL) (*Zalophus californianus*) haul-out in large numbers at sites where fecal contamination and pathogen pollution can be significant; therefore it is possible that these marine mammals are exposed to and infected with protozoan parasites. *Cryptosporidium parvum* and *Giardia duodenalis* have been detected in the feces of California sea lions recovered from a marine mammal rehabilitation facility located in the northern California coastal area (15), documenting for the first time the presence of infections with these potentially zoonotic pathogens. Although this suggests that California sea lion haul-out sites may serve as a source of parasite contamination, the public health implications are still not clear since the assemblages of the *G. duodenalis* detected in sea lions have not been determined. Understanding the role of CSL in the contamination of marine environments and the zoonotic potential of the protozoa shed in their feces is important because these marine mammals can be found living close to beaches that are heavily used by humans for recreation and seafood harvest. Unfortunately, the chances of detecting a positive fecal sample in the field can be difficult because fecal shedding of oocysts and cysts is usually only high after acute infection and can then be intermittent.

In this study, wild mussels were used as bioindicators of fecal protozoa in environmental samples. This approach has merit due to the high filtration
rate of mussels that can filter over 2 L of water/hr/shellfish (16), which helps overcome dilution limitations of direct water testing. Shellfish have been used as bioindicators of pathogen pollution in nearshore waters in previous studies (5, 13, 17) because pathogenic microorganisms that occur in marine environments may be filtered by the gills during feeding and become concentrated in the digestive tract/glands of the mollusk (18). Mussels are also frequently found at CSL haul-out sites and, therefore, may be exposed to fecal material. Thus, molecular identification of Cryptosporidium and Giardia in these mussels could provide information about the possible role of CSL in the transmission of protozoan parasites to the marine environment.

Previous studies have found evidence of Cryptosporidium oocysts and Giardia cysts in marine environments by DNA amplification of parasite sequences in the tissues of filter-feeding bivalves that serve as prey for both sea otters and some humans in coastal California (13, 19-21). Host-specific and anthropozoonotic Cryptosporidium genotypes have been recovered previously from marine shellfish in California (22); however whether these oocysts were shed by terrestrial animals and/or marine mammals is unclear.

The objectives of this study were 1) to test for the presence of Cryptosporidium oocysts and Giardia cysts in mussels at two distinct coastal areas: a) land runoff plume sites and b) near CSL haul-out sites, as well as in CSL feces, and 2) genotype protozoal DNA detected in California sea lion
feces and mussels (*Mytilus californianus*). We hypothesized that the same
genotypes found in terrestrial animals would be present in mussels and CSL
feces if pathogen pollution was flowing from land to sea. The results of this
study will contribute to our understanding of the fate and transport of
*Cryptosporidium* and *Giardia* parasites in nearshore marine environments,
and the possible role of CSL as a nearshore host for these parasites.

Methods

The presence of the protozoan parasites *Cryptosporidium* spp. and
*Giardia* spp. was screened in mussels (*Mytilus californianus*) and the feces of
California sea lions (*Zalophus californianus*) collected from the wild. Specific
sampling sites were selected based on accessibility and safety for boat-
based and ground work, and locations where freshwater runoff and sea lion
haul-out sites are distinctly separated. Collection dates were based on
periods of low tide, surf, and swell. Samples were collected during the wet
season (December to May) and dry season (June to November) (23) for 2
years (2011 to 2013). A wet season was identified as the period from when
the seasonal rivers began discharging to the ocean until 30 days after the
river mouth closed; otherwise the season was considered dry.

Sample collection:
California sea lion (CSL) feces were collected from haul-out sites (rookeries where the CSL rest) located at Año Nuevo Island near Pescadero, Point Lobos State Reserve near Monterey, and White Rock near Cambria, in California (Figure 1). Feces to be analyzed for *Cryptosporidium* oocysts and *Giardia* cysts were collected with a tongue depressor and then placed in 50 ml conical tubes that were placed in plastic bags labeled with the site name and date of collection. All samples were placed in ice chests and transported within 24 hours to the UC Davis laboratory, where they were stored at 4°C and processed within 48 hours of collection.

Mussels were collected near two possible sources of pathogen pollution, adjacent to two freshwater sources (Carmel River in Monterey area and Santa Rosa Creek in Cambria area, Fig. 1), and near the two CSL haul-out sites (Point Lobos State Reserve in Monterey area and White Rock in Cambria area) described above. At each site, at least 40 mussels were collected during each sampling effort for the 2 year sampling period. Mussels were collected during low tide, and placed in plastic bags labeled with the site name, date of collection and number of mussels collected. Samples were shipped on ice to UC Davis for pathogen analysis within 24 hours.

Fecal sample processing:

Five g of CSL fecal sample, or the whole sample if it weighed less than 5 g (15), were processed as previously described in Dabritz et al. (24) with...
the following modifications. The feces were homogenized in Tween 0.1% solution and the tubes were centrifuged at 1000 G for 20 minutes. With a smooth movement, the supernatant was removed, and with a loop the surface of the pellet was transferred to a well of a 3-well slide, as described in Marks et al. (25). Within 12 hours, the slides were stained by the direct fluorescent antibody (DFA) technique using Waterborne kit reagents (Aquaglo™ G/C Direct, Waterborne Inc, New Orleans, Louisiana) per the manufacturer’s instructions and according to Environmental Protection Agency (EPA) Method 1623 (17). Slides were stained with fluorescein isothiocyanate (FITC) and 4, 6-diamidino-2-phenylindole (DAPI), and examined for protozoa using an Axioscop epifluorescent microscope.

Organisms were visualized at 200x magnification and identification confirmed at 400x magnification (17). Cryptosporidium oocysts were identified as ~5 μm spheres outlined in apple green and often with a mid-line seam, whereas Cryptosporidium andersoni/muris-like were identified as 5x7 μm elliptical organisms (17). Giardia cysts were also apple green but oval and 9-14 μm long (17). All slides were read by the same experienced microscopist and confirmed by a second microscopist. Any DFA-positive samples were concentrated with immunomagnetic separation (IMS) (Dynal Biotech, Olso, Norway), as previously described by Miller et al. (17) with some modifications. Five loops filled with fecal sample were processed, the beads-parasite complexes dissociation step with 0.1 N HCL was omitted and
the concentrated samples were placed in a 1.5 ml microcentrifuge tube instead of on to a DFA slide. The samples were then subjected to DNA extraction and conventional PCR (described in detail below) for confirmation and sequence analysis of *Cryptosporidium* and *Giardia*.

Hemolymph sample processing

Hemolymph samples were taken from 30 individual mussels from each site. The hemolymph collection methods, DNA extraction, amplification and sequencing methods were as described by Miller et al. (26). The cell pellet homogenized in 100 µl of supernatant was subject to DNA extraction adapting the Qiagen DNA mini kit® manufacturer instructions (described below) within 24 hours of extraction. Hemolymph and DNA were stored at -20°C. Hemolymph samples were not processed by DFA methods because hemocytes tend to autofluoresce; thus making oocyst visualization difficult (22).

Mussel tissue sample processing

Tissues of 30 mussels per site per sampling event were scraped and blended to obtain a pool of mussel tissue homogenate. Mussel homogenate was then sieved using 100 µm cell strainers to obtain three aliquots of 4 g each per sample per site per sampling event. The samples were centrifuged at 1000 G for 15 minutes and the supernatant was removed from the tube.
Detection of Cryptosporidium oocysts and Giardia cysts in the pellets of mussel homogenate involved concentration by IMS and quantification by DFA technique as described above, except that the beads-parasite complex dissociation step was included as described in Miller et al. (17). The parasite dissociation steps were repeated once. Parasite-positive samples were then subject to DNA extraction and conventional PCR for confirmation and sequencing of Cryptosporidium and Giardia as described below.

DNA extraction protocol

A modification of Miller et al. (22) protocol was followed for DNA extraction from a 100 µl maximum pellet of hemolymph and DFA positive or suspect CSL feces and mussel tissue homogenate. Proteinase K (40 µl) was added to the sample and kept at 56°C overnight for sample digestion. Using the QIAGEN DNA Mini Kit tissue protocol (QIAGEN Inc., Valencia, CA), the sample was bound to a QIAamp column, washed, and the DNA eluted with 50 µl of 95°C 10% buffer AE. Extracted DNA was stored at -20°C until PCR analysis.

Conventional PCR protocols

PCR techniques for detection of Cryptosporidium spp. DNA in hemolymph, DFA-positive CSL feces and DFA-positive mussel homogenate were based on two genes used for molecular characterization: 18rRNA gene
To initially screen samples, a 298 bp DNA fragment was amplified using a modification of the Morgan 18S rRNA PCR protocol. This assay was chosen as a screening tool as a previous study showed that the Morgan protocol was more sensitive though less specific than the Xiao 18S rRNA assay (17, 26).

PCR reactions contained 5 µl (1x) 10X PCR buffer with 1.5 mM MgCl₂, 3 µl (3 mM) of additional MgCl₂, 1 µl (200mM) dNTPs, 0.2 µl (0.4µg/ml reaction) 10% bovine serum albumin (BSA), 0.5 µl (200nM) of each primer at a concentration of 20pmol/µl, 0.25 µl (1.25 U) HotStar Taq plus polymerase and 3 µl DNA for hemolymph and 5 µl for CSL feces, and PCR-grade water in a 50 µl total volume. Amplification conditions for the PCR reactions started with 95°C for 5 min, followed as previously described in Morgan et al. (27). Amplified PCR products were combined with 2 µl of Blue/Orange loading dye (Promega Corporation, Fitchburg, Wisconsin) and loaded into a 1.5-2% gel stained with ethidium bromide (1.5 µl) for electrophoresis.

To further characterize samples that yielded a positive result using the Morgan 18S rRNA assay, a 850 bp DNA segment was amplified using a modified Xiao 18S rRNA nested PCR protocol (28, 29). PCR reactions contained 5 µl (1x) 10X PCR buffer with 1.5 mM MgCl₂, 12 µl (6mM) and 6 µl (3mM) MgCl₂ for round 1 and 2 respectively, 1 µl (0.2 mM) dNTPs, 0.2 µl (0.4 µg/ml reaction) 10% bovine serum albumin (BSA), 0.5 µl (200nM) of
each primer at a concentration of 20pmol/µl, 0.25 µl (1.25 U) HotStar Taq plus polymerase, 3 µl and 5 µl DNA for round 1 and round 2 respectively, and PCR-grade water in a 50 µl total volume. Amplification conditions for the PCR reactions started with 95°C for 5 min, followed as previously described settings described in Xiao et al. (28).

To allow for sequence analysis at a second locus, Xiao 18S-positive samples were further processed to amplify a 550 bp DNA fragment using a modified Spano COWP PCR protocol (30). All PCR reactions contained 5 µl (1x) 10X PCR buffer with 1.5 mM MgCl₂, 3 µl (3mM) MgCl₂, 1 µl (0.2 mM) dNTPs, 0.2 µl (0.4 µg/ml reaction) 10% bovine serum albumin (BSA), 1.5 µl (200nM) of each primer at a concentration of 20pmol/µl, 0.25 µl (1.25 U) HotStar Taq plus polymerase and 3 µl DNA, and PCR water in a 50 µl total volume. Amplification conditions for the PCR reactions started with 95°C for 5 min, followed as previously described by Spano et al. (30).

Genotype analysis of *Giardia* spp. in hemolymph, DFA-positive CSL feces, and DFA-positive mussel homogenate was based on two loci used for molecular characterization: GDH gene (31) and beta-giardin gene (32). A 432 bp fragment was amplified using a modified GDH PCR protocol (31). The PCR reactions contained 5 µl (1x) 10X PCR buffer with 1.5 mM MgCl₂, 1 µl (0.2 mM) dNTPs, 0.2 µl (0.4 µg/ml reaction) 10% bovine serum albumin (BSA), 1.25 µl (25pmol/reaction) of each primer at a concentration of 20pmol/µl, 0.25 µl (1.25 U) HotStar Taq plus polymerase and 3 µl and 1 µl...
DNA for round 1 and round 2, respectively, and PCR water in a 50 µl total volume. Amplification conditions for the PCR reactions started with 95°C for 5 min, followed as previously described by Read et al. (31).

For GDH-positive samples, a second DNA locus was targeted by amplifying a 384 bp DNA segment using a modified beta-giardin semi-nested PCR protocol (32). All PCR reactions contained 5 µl (1x) 10X PCR buffer with 1.5 mM MgCl₂, 1 µl (0.2 mM) dNTPs, 0.2 µl (0.4 µg/ml reaction) 10% bovine serum albumin (BSA), 0.5 µl (200 nM) of each primer at a concentration of 20pmol/µl, 0.25 µl (1.25 U) HotStar Taq plus polymerase and 3 µl and 1 µl DNA for round 1 and round 2, respectively, and PCR water in a 50 µl total volume. Amplification conditions for the PCR reactions started with 95°C for 5 min, followed by 33 cycles with thermocycler settings as previously described by Caccio et al. (32). The Primer sequences for all PCR protocols used in this study are listed in Table 1.

Samples with amplified DNA bands matching the sizes described above for each PCR protocol were purified using either ExoSAP-IT (Affymetrix Inc., Cleveland, Ohio) or QIAquick PCR (Qiagen Inc., Chatsworth, California) purification kits following the manufacturer instructions, and the samples were submitted to the UC core DNA Sequencing Facility for sequence analysis.

Phylogenetic analysis
The ends of the forward and reverse DNA sequences were trimmed and then aligned using the Codon Code aligner software (Codon Code Corporation, Massachusetts). The amplified DNA sequences identified in CSL feces, mussel tissue homogenate and mussel hemolymph were compared with Genbank reference sequences for Cryptosporidium and Giardia using Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Clustal X (33) software. The phylogenetic analysis was inferred based on Kimura 2-parameters distance estimates with 1000 bootstrap replicates, and trees were constructed by using the Neighbor-Joining algorithm (34), implemented in the MEGA 5.0 software (35).

Preliminary spiking studies were performed to evaluate assay sensitivity for parasite detection (data not shown). Previous CSL fecal samples and individual mussel hemolymph spiking experiments with oocysts of C. parvum Iowa isolate (Harley Moon) passaged through calves, and gerbil-passaged cysts of G. lamblia human isolate H-3 obtained from Waterborne Inc (New Orleans, LA) showed that the minimum detection in 5 grams of feces was either 10 oocysts and 10 cysts by DFA. For CSL fecal samples tested by PCR, the minimum detection limit was 10 oocysts by Morgan 18S rRNA, 10 oocysts by Xiao 18S rRNA, 100 oocysts with Spano COWP and 1 cyst by GDH. For mussel hemolymph samples tested by PCR, the minimum detection limit was 1 oocyst or 1 cyst for all of primers used in this study.
Results

Over the 2-year duration of the study, a total of 303 individual CSL fecal samples were collected and tested, of which 133 were collected from White Rock (86 during the dry season and 47 during the wet season), 113 were collected from Point Lobos State Reserve (58 during the dry season and 55 during the wet season), and 57 from Año Nuevo Island (41 during the dry season and 16 during the wet season). Table 2 shows the prevalence of samples per site with oocysts and cysts that had the morphologic features characteristic of *Cryptosporidium* and *Giardia*, respectively, and which were DFA positive. *Cryptosporidium* oocysts were detected in 8.3% (9.3% during the dry season and 6.4% during the wet season) of individual CSL fecal samples collected from White Rock; 15% of individual fecal samples (6.9% during the dry season and 23.6% during the wet season) from Point Lobos; and 3.5% (2.4% during the dry season and 6.3% during the wet season) individual fecal samples collected on Año Nuevo island. *Giardia* cysts were detected in CSL fecal samples collected from White Rock, with a prevalence of 3.0% (3.5% during the dry season and 2.1% during the wet season).

Counts in *Cryptosporidium* and *Giardia*-positive CSL fecal samples detected by DFA without IMS were under 10 oocysts/cysts per sample, except for three samples, one collected in White Rock (>100 oocysts) and the other sample collected on Año Nuevo island (98 oocysts) both during the
dry season, and the third one collected in Point Lobos during the wet season. Cryptosporidium and Giardia DNA was not identified by IMS in combination with PCR in any of the CSL fecal samples.

Over the 2 year study, hemolymph was extracted from 961 mussels, of which 479 were collected during the dry season and 482 were collected during the wet season (Table 2). No hemolymph samples yielded positive results for Cryptosporidium DNA by PCR using the Morgan 18S rRNA (298 bp target) and Xiao 18S rRNA nested (850 bp) PCR protocols. Table 2 shows the proportion of hemolymph samples that were positive for Giardia DNA per site by PCR.

Giardia spp. DNA was identified by PCR amplification of GDH sequences that were confirmed by sequence analysis in 0.4% (4/961) of individual hemolymph samples. Only one of the strongest GDH positive samples (432 bp target) was also positive by beta-giardin PCR (384 bp target), a protocol that has been less sensitive previously. Of the 68 PCR products of amplification with GDH primers, Blast searches in GenBank revealed four DNA sequences (isolates HW1, HW4, HS71 and HC216) that closely matched reference sequences for Giardia intestinalis, 6 DNA sequences that were more closely related to non-Giardia microorganisms, and 48 DNA sequences that were mixed. Of the three DNA sequences found to closely match the reference sequences for Giardia intestinalis, two of them (HW1 and HW4) were detected in hemolymph from mussels collected on September 23, 2017.
from White Rock during the dry season. Isolate HW4 was 96% similar to *G. intestinalis* assemblage D (GenBank accession number GdD_EF507636) by Blast and Clustal X analysis, with only 13 mismatching nucleotides out of a total of 421 nucleotides. The other GDH sequence (isolate HW1) was 97% similar to *G. intestinalis* assemblage C (GenBank accession number AB569390 and U60985) by Blast and Clustal X, with only nine mismatching nucleotides of a total of 425 nucleotides. This sample was also positive by beta-giardin PCR (isolate HW1), and the DNA sequence was 99% similar to *G. intestinalis* assemblage D (GenBank accession number AY545647, HQ538708, HQ538709 and FJ009205) according to both Blast and Clustal X, with only one mismatching nucleotide of a total of 313 nucleotides. The third GDH-amplified DNA sequence (isolate HS71) that closely matched reference *G. intestinalis* was detected in the hemolymph of mussels collected in Santa Rosa Creek during the wet season. The sequence similarity to *G. intestinalis* assemblage B (GenBank accession numbers AY178749, AY178750, HM134215 and EU594666) was 100% by Blast and 99.8% by Clustal X, with only one mismatching nucleotide of a total of 411 nucleotides. The fourth GDH-amplified DNA sequence (isolate HC216) that closely matched reference *G. intestinalis* was detected in the hemolymph of a mussel collected near Carmel River during the wet season. This sequence was 100% similar to *G. intestinalis* assemblage D (GenBank accession numbers
F507619, JX448631 and U60986) by both Blast and Clustal X, with no mismatching nucleotides of a total of 407 nucleotides.

*Giardia* spp. DNA identified in hemolymph samples were uploaded to the GenBank database. Figures 2 and 3 show the phylogenetic analyses of isolates HW1 (Assemblage C, GenBank KF294079) and HW4 (Assemblage D, GenBank KF294080) obtained from mussels at the White Rock study site, as well as isolates HS71 (Assemblage B, GenBank KF294081) and HC216 (Assemblage D, GenBank KF294078) detected in mussels from Santa Rosa Creek and Carmel, CA respectively. Isolates HW1, HW4, HS71 and HC216 were not all exact matches to reference sequences but were classified within the *Giardia* clade, with HW1 most closely related to *G. duodenalis* assemblage C based on the GDH locus (Figure 2) and located in the same clade as *G. intestinalis* assemblage D based on the beta-giardin locus (Figure 3). As shown in Figure 2, HW4 and HC216 were most closely related to *G. duodenalis* assemblage D, and HS71 was most closely related to *G. duodenalis* assemblage B with the GDH locus.

Discussion

In this study, hemolymph and tissue homogenates collected from filter-feeding mussels and fecal samples from California sea lions at two study sites along the central coast of California were tested for *Cryptosporidium* and *Giardia* spp. Notably, DNA of *Giardia duodenalis*
assemblages B and D were detected in the hemolymph of mussels located near land runoff sites, at Santa Rosa Creek and Carmel River, and assemblages C and D DNA were detected in hemolymph from mussels collected near the CSL haul-out site at White Rock. In addition, the presence of *G. duodenalis* assemblage B, which is known to have zoonotic potential, provided evidence of a potential health risk to beachgoers and surfers, particularly during the wet season when river runoff is high due to rainfall. Rainfall can result in mobilization of fecal material from land and subsequent discharge into rivers, increasing the concentration of fecal pathogens and exposure of both mussels and mammals, including humans, to these pathogens (29, 36). The *G. duodenalis* assemblages C and D that were identified in mussels collected at the CSL haul-out site can also have implications for public health, as they have been shown to be infectious to humans (37), though they appear to be most commonly shed by domestic and wild canids. Although *Giardia*-like cysts were observed in CSL feces in this study, their genotypes were not identified so the source of the *G. duodenalis* DNA in the mussels collected at haul-out sites could not be confirmed.

The molecular characterization of *Giardia* in mussels in this study affirms the usefulness of testing filter-feeding shellfish, such as mussels, that can concentrate protozoan parasites and other pathogens in aquatic environments (21, 22, 38-41). Unlike the present study, however, the
predominant protozoan parasite reported by previous authors was

*Cryptosporidium*, most notably *C. parvum*. In our study, *Giardia* was

molecularly identified and characterized from mussel hemolymph, whereas

*Cryptosporidium* was not confirmed using PCR methods with sequence

analysis in any of the mussel hemolymph samples collected. The low

prevalence of *Giardia* and lack of detection of *Cryptosporidium* in the

hemolymph samples tested in this study might be explained by the fact that

the concentration of cysts and oocysts coming from river outflow was diluted

as fecally contaminated water was discharged into the sea; so despite the

ability of mussels to concentrate these parasites stages, the levels were

often lower than our detection limits. The low number of positive samples in

mussels could also be due to the fact that both wet seasons during which

mussels were sampled were low in total rainfall, and our sampling occurred

late in the wet season (around April in 2012). Another possible explanation

for the low prevalence of these protozoan parasites in the hemolymph

samples is that prior studies have demonstrated that bivalves depurates the

pathogens concentrated from surrounding water over the following days or

weeks (17). Most of our bivalve sampling did not occurred immediately after

storm events when bivalves would be most likely to retain higher pathogen

concentrations and are most useful as bioindicators (42).

In the present study, there was a discrepancy between the sequence

analysis results obtained with the GDH and the beta-giardin PCR protocols
for the amplification of Giardia DNA. Analysis at the GDH locus revealed that Isolate HW1 was related to G. duodenalis assemblage C; conversely the beta-giardin locus suggested that this isolate was related to G. duodenalis assemblage D. Similar mixed multi-locus genotyping results were reported in three other studies (43-45) and deserve further molecular epidemiologic investigation. Additionally, amplified DNA sequences of isolates HW4, HS71 and HC216 were detected using the GDH PCR protocol but not with the beta-giardin PCR protocol. Nantavisai et al. (46) spiked stool samples with known numbers of Giardia cysts to assess the sensitivities of different Giardia PCR methods and obtained better recovery efficiencies by the GDH locus compared to the beta-giardin locus.

Very few studies have investigated the presence of Cryptosporidium oocysts and Giardia cysts in fecal samples from CSLs in the wild. In a previous study, Deng et al. (15) identified and molecularly characterized C. parvum and Giardia duodenalis in fresh fecal samples collected from stranded CSLs in a marine animal recovery and rehabilitation facility. These CSL could have been more exposed to infection as they were in contact with humans and other terrestrial animals, and also more susceptible due to stress or illness, causing the CSL to shed sufficient oocysts and cysts for the detection of DNA by PCR. In the present study, CSL fecal samples came from haul-out sites where the animals were apparently healthy and free-ranging; however, under these circumstances feces could not be collected.
immediately after defecation which could explain the low counts detected for both protozoa. Another possible explanation for the low counts detected in our study is that the CSL fecal samples were collected approximately every 3 months which could have reduce the likelihood of detecting higher counts of oocysts and cysts as oocysts and cyst has an intermittent shedding pattern in feces (8). Nonetheless, fresh samples were collected and the presence of oocysts and cysts with the morphologic features characteristics of *Cryptosporidium* spp. and *Giardia* spp. in fecal samples from wild CSLs at White Rock and Point Lobos, along with the identification of *Giardia* DNA in hemolymph of mussels collected at the CSL haul-out site at White Rock. Unfortunately, it was not possible to confirm by genotype analysis the isolates of both *Cryptosporidium*-like oocysts and *Giardia*-like cysts identified in the feces of CSL. The lack of amplification of DNA sequences for *Cryptosporidium* and *Giardia* could have been due to the low number of oocysts and cysts detected in the fecal samples. The detection of oocysts and cysts with the morphologic features characteristics of *Cryptosporidium* spp. and *Giardia* spp. in fecal samples from wild CSLs suggests that CSLs may serve as a source of these protozoan parasites and might be contributors of the parasite load discharged in their natural marine environment. Further studies are needed to better understand the role of CSL as host of *Cryptosporidium* and *Giardia* in marine environs including genotyping and viability studies of protozoa detected in CSL feces.
Conclusions

In summary, detection of *G. duodenalis* assemblages B, C and D in the hemolymph of mussels sampled near freshwater runoff and CSL haul-out sites in coastal California confirmed the usefulness of testing shellfish that are capable of concentrating protozoans from marine environments and is consistent with terrestrial sources for these fecally-transmitted, waterborne parasites flowing from land to sea. The detection of *G. duodenalis* assemblage B in mussels collected near land runoff in Carmel is noteworthy as this assemblage is known to be infectious to humans thus may pose public health risks. Detection of *G. duodenalis* assemblages C and D in mussels collected in CSL haul-out sites provides evidence of their presence in the waters surrounding haul-out sites; Overall, the results of this study support the hypothesis that the same genotypes found in terrestrial animals can be found in mussels, however further molecular studies are needed to ascertain whether CSL fecal samples have the same genotypes found in terrestrial animals.

Acknowledgements

We acknowledge the financial support received from the “Presidente de la República” Chilean scholarship, the Central Coast Long-term Environmental Assessment Network (CCLEAN) (grant #: 06-076-553) from...
the California State Water Board to the City of Watsonville, the National Science Foundation (NSF) Ecology of Infectious Disease Grant Program (grant #: OCE-1065990), UC Davis Graduate Student Support Program (GSSP) and UC Davis Graduate group in Comparative Pathology Block Grant. Thanks are due to the field teams that supported collections of mussels, and specifically acknowledge Tim Tinker, Joe Tomoleoni, Jim Webb, Ben Weitzman, Don Canestro, Miles Daniels, Mark Kocina, Colin Krusor, Zach Randell and Matt Smith. Collection of mussels from the sites in Cambria was facilitated through the University of California Ken Norris Rancho Marino Reserve. Thanks are also due to Chuck Bancroft, Sean James and Erik Abma, and Patricia Morris for facilitating collection of CSL fecal samples at Point Lobos State Reserve and Año Nuevo Island, respectively. We are also grateful to the laboratory team that assisted in the processing of the mussels, and specifically acknowledge Beatriz Aguilar, Andrea Packham, Terra Berardi, Heather Fritz, Leopoldo Guerrero, Kaitlyn Hanley, Claudia Llerandi, Lauren Michaels and Anna Naranjo.
References


25. **Marks SL, Hanson TE, Melli AC.** 2004. Comparison of direct immunofluorescence, modified acid-fast staining, and enzyme


and PCR methods for detection of *Giardia duodenalis* in stool specimens.

Table 1. Nucleotide sequence of polymerase chain reaction (PCR) primers used to detect DNA of *Cryptosporidium* and *Giardia* species in mussel hemolymph and tissue homogenates, and sea lion feces.

<table>
<thead>
<tr>
<th>Amplification target</th>
<th>PCR protocol</th>
<th>Primer</th>
<th>Primer sequence (5’-3’)</th>
<th>Length of final PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cryptosporidium</em> spp.</td>
<td>Morgan 18S rRNA&lt;sup&gt;1&lt;/sup&gt;</td>
<td>18SF</td>
<td>AGTGACAAGAAATAACAATACAGG</td>
<td>298</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18SR</td>
<td>CCTGCTTTAAGCACTCTATAATTTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xiao 18S rRNA&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>Xiao EF</td>
<td>TTCTAGAGCTAATACATGCAG</td>
<td>819-825</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xiao ER&lt;sup&gt;3&lt;/sup&gt;</td>
<td>CCCATTTCCCTCGAACAGGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xiao IF</td>
<td>GGAAGGGTTGTATTTATAGATAAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xiao IR</td>
<td>AAGGAGTAAAGGAAACCTCCA</td>
<td></td>
</tr>
<tr>
<td><em>Giardia</em> spp.</td>
<td>Spano COWP&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Cry-15 EF</td>
<td>GTAGATAATGGAAGATTTG</td>
<td>550</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cry-9 ER</td>
<td>GGACTGAAATACAGGCATTCTTG</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Morgan 18S rRNA: AGTGACAAGAAATAACAATACAGG, CCTGCTTTAAGCACTCTATAATTTC
<sup>2</sup> Xiao 18S rRNA: TTCTAGAGCTAATACATGCAG
<sup>3</sup> Xiao 18S rRNA: CCCATTTCCCTCGAACAGGA
<sup>4</sup> Spano COWP: GTAGATAATGGAAGATTTG, GGACTGAAATACAGGCATTCTTG
<table>
<thead>
<tr>
<th>PCR Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry-12 IF</td>
<td>CCAGATGGATTCAGATTATTGGG</td>
</tr>
<tr>
<td>Cry-14 IR</td>
<td>CTATCTTTTACAACCCCGATGGGC</td>
</tr>
<tr>
<td>GDHeF</td>
<td>TCAACGTYAAYCGYGGYTTCCGT</td>
</tr>
<tr>
<td>GDHiF</td>
<td>CAGTACACTCYGCTCTCGG</td>
</tr>
<tr>
<td>GDHiR</td>
<td>GTRRTCCTTGACATCTCC</td>
</tr>
<tr>
<td>Beta-giardin6</td>
<td>Caccio G7 eF AAGCCCGACGACCTACCCGCAGTGCTG</td>
</tr>
<tr>
<td></td>
<td>Caccio G759 eIR GAGGCCGCCCTGGATTTGAGCACGAC</td>
</tr>
</tbody>
</table>

1. Morgan et al. (27)
2. Xiao et al. (28)
3. Xiao et al. (29)
4. Spano et al. (30)
5. Read et al. (31)
6. Caccio et al. (32)
Table 2. Prevalence of *Cryptosporidium* and *Giardia*-positive California sea lion (CSL) fecal samples, individual mussel hemolymph samples, and pooled mussel tissue homogenate collected at sample sites along the central California coast.

<table>
<thead>
<tr>
<th>Site</th>
<th>Season</th>
<th>CSL feces</th>
<th>Hemolymph</th>
<th>Mussel homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Crypto</td>
<td>Giardia</td>
<td>Crypto</td>
</tr>
<tr>
<td>White Rock</td>
<td>Dry</td>
<td>9.3% (8/86)</td>
<td>3.5% (3/86)</td>
<td>0% (0/118)</td>
</tr>
<tr>
<td></td>
<td>Wet</td>
<td>6.4% (3/47)</td>
<td>2.1% (1/47)</td>
<td>0% (0/120)</td>
</tr>
<tr>
<td>Santa Rosa Creek</td>
<td>Dry</td>
<td>NA⁴</td>
<td>NA⁴</td>
<td>0% (0/121)</td>
</tr>
<tr>
<td></td>
<td>Wet</td>
<td>NA⁴</td>
<td>NA⁴</td>
<td>0% (0/120)</td>
</tr>
<tr>
<td>Point Lobos</td>
<td>Dry</td>
<td>6.9% (4/58)</td>
<td>0% (0/58)</td>
<td>0% (0/121)</td>
</tr>
<tr>
<td></td>
<td>Wet</td>
<td>23.6% (13/55)</td>
<td>0% (0/55)</td>
<td>0% (0/90)</td>
</tr>
<tr>
<td>Carmel River</td>
<td>Dry</td>
<td>NA⁴</td>
<td>NA⁴</td>
<td>0% (0/119)</td>
</tr>
<tr>
<td></td>
<td>Wet</td>
<td>NA⁴</td>
<td>NA⁴</td>
<td>0% (0/152)</td>
</tr>
<tr>
<td>Año Nuevo Island</td>
<td>Dry</td>
<td>2.4% (1/41)</td>
<td>0% (0/41)</td>
<td>NC⁵</td>
</tr>
</tbody>
</table>

⁴ NA: Not available
⁵ NC: Not calculated
<table>
<thead>
<tr>
<th></th>
<th>Wet</th>
<th>0% (0/16)</th>
<th>NC⁵</th>
<th>NC⁵</th>
<th>NC⁵</th>
<th>NC⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total collected</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry</td>
<td>7.0% (13/185)</td>
<td>1.6% (3/185)</td>
<td>0% (0/479)</td>
<td>0.4% (2/479)</td>
<td>0% (0/39)</td>
<td>0% (0/39)</td>
</tr>
<tr>
<td>Wet</td>
<td>14.4% (17/118)</td>
<td>0.9% (1/118)</td>
<td>0% (0/482)</td>
<td>0.4% (2/482)</td>
<td>0% (0/15)</td>
<td>0% (0/15)</td>
</tr>
<tr>
<td>Overall</td>
<td>9.9% (30/303)</td>
<td>1.3% (4/303)</td>
<td>0% (0/961)</td>
<td>0.4% (4/961)</td>
<td>0% (0/54)</td>
<td>0% (0/54)</td>
</tr>
</tbody>
</table>

¹Positive samples detected by direct fluorescent antibody (DFA)

²Positive samples detected by polymerase chain reaction (PCR) methods and confirmed by sequence analysis

³IMS and DFA-positive aliquots of 4g of mussel tissue homogenate

⁴Not applicable as this site is not a CSL haul-out site

⁵Not collected due to low numbers of specimens in the area and unsafe sampling conditions
Figure 1. Locations of mussel and California sea lion (CSL) fecal sample collection sites along the central California coast with (□) representing CSL haul-out sites, (●) representing land runoff sites, and (★) representing Giardia DNA positive mussel hemolymph. Map created using Quantum GIS version (QGIS) 1.8-Lisboa open source software (http://qgis.osgeo.org).
Figure 2. Phylogenetic analysis of *Giardia duodenalis* sequences detected in hemolymph from mussels based on the nucleotide sequence at the GDH locus and using a neighbor joining Kimura 2-parameter method. (GenBank reference accession numbers are shown in parenthesis).

*Isolates detected in hemolymph from mussels in this study.*
Figure 3. Phylogenetic analysis of *Giardia duodenalis* sequences detected in hemolymph from mussels based on the nucleotide sequence at the beta-giardin locus and using neighbor joining a Kimura 2-parameter method. (GenBank reference accession numbers are shown in parenthesis).

*Isolate detected in hemolymph from mussels in this study.*